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13. ABSTRACT *(Maximum 200 Words)*

The goal of this proposal was to quantify the extent to which human prostate cancer cells vary in their proteolytic proteasome-associated activities, and to evaluate the role of the proteasome in the response of prostate cancer cells to hyperthermia and/or radiotherapy. Cell lines have been shown to vary considerably in their proteasome activity. Both irradiation and hyperthermia inhibit activity, but by different mechanisms. Further support has been obtained for the hypothesis that hyperthermia acts through HSP90 activation. We have shown that 26S, but not 20S activity, is affected by heat, while both are affected by irradiation. New techniques have been developed to look at this further.

Many pathways are affected by proteasome inhibition. Hyperthermia has been shown to unexpectedly decreased androgen receptor expression and result in androgen-independent growth of LnCaP cells. Radiation and heat treatment prevent NF-kB activation, although high radiation doses can circumvent ongoing proteasome inhibition to paradoxically activate NF-kB. Hyperthermia blocks this activation, suggesting a mechanism for hyperthermia-induced radiosensitization, since NF-kB is a known survival factor for cells. A pathway involving activation of caspase 3, degradation of the repair enzyme DNA-PKcs, and inhibition of repair has been shown to be unlikely.

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Research Accomplishments to Date

Introduction:

The aim of this IDEA Award was to investigate the novel hypothesis that the proteasome has significant roles in the response of prostate cancer cells to hyperthermia and/or irradiation. Recent evidence indicates critical roles for the 26s proteasome in selectively regulating many vital cell processes, including cell cycle progression, oxidative stress, DNA repair, and cell death. This proposal is novel in that the roles of the proteasome in the biology and therapy of cancer have been the subject of very few investigations and there are almost no reports on how their activities might influence the response of prostate cancer to therapeutic intervention. We postulated that proteasome activity would vary between prostate cancer cell lines, that hyperthermia and/or radiation would influence proteasome activity, and that this would influence the expression of cell death/survival molecules following treatment in a manner that could determine therapeutic outcome. We further suggested that the 26s proteasome might represent a novel potential target for prostate cancer therapy; one that is, as yet, unexplored.

Progress will be considered under the headings of the original stated tasks.

Body of the Report:

Task 1. To quantify proteasome activity in human prostate cancer cells and its modification by hyperthermia and/or radiotherapy.

Proteasome-associated chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activity has been measured in PC3, LnCaP, DU-145, and a newly isolated human prostate line LAPC4. As predicted, considerable variation was found suggesting that the proteasome might determine the molecular phenotype of the cell, and as a result, determine tumor behavior and the response to therapy. We had hoped to extend these studies using number of other cell lines that have been isolated at UCLA and grow in immune deficient mice, but for reasons that are unclear we have had problems in culturing these *in vitro*. As an alternative approach, we are intend to extend our investigation of LnCaP by testing clones of cells that have become androgen independent and that we have been involved in characterizing [Tso, 2000]. Cellular and molecular properties of these androgen-independent variants were characterized both in vitro and in vivo and compared with those of their parental androgen-dependent cells. In contrast to the poorly tumorigenic parental LNCaP cells, these lines proved highly tumorigenic, exhibiting invasive and metastatic characteristics in intact and castrated mice and in female mice within a short period of 3 to 4 weeks. In addition, these clones develop resistance to irradiation and cytotoxic drugs. Because they are isogenic with the parent LnCaP cell line, they will enable us to further investigate the link between proteasome activity, loss of androgen responsiveness, and radioresistance.

The response of PC-3, LnCaP, and DU-145 to heat has been examined. Hyperthermia at 42 or 44 degrees for 30 minutes caused apoptosis and radiosensitization in all three cell lines and decreased 26s proteasome activity significantly to about 40% of untreated control cells, as assessed by proteolysis of specific fluorogenic substrates (chymotrypsin-like activity was decreased to 36.2 ± 3% (PC-3), 33.4 ± 8.4% (DU-145) and 45 ± 3.4% (LnCaP)). Inhibition of 26 proteasome activity was independent of transcriptional activity, as judged by inhibitor studies. Interestingly, 20s proteasome activity was unaffected. This suggests that simple blocking of the proteasome by oxidised proteins is unlikely to be the mechanism of inhibition and that processing of proteins by 19S regulatory subunits that feed proteins into the catalytic core are the target. We had hypothesized that HSP90 might play a role in heat-induced proteasome inhibition. We have strengthened this view by finding that the response to heat correlated with HSP90 induction, as assessed by PCR, in a temporal and temperature-related manner, but not with HSP70 or HSP27 expression. Also, recombinant HSP90 blocked the activity of purified proteasomes. This is taken

as support for our hypothesis that HSP90 is an inhibitor of proteasome activity and mediating at least some of the inhibition we have observed.

We had previously shown that proteasome inhibition would affect degradation of the NF-kB inhibitor, IkBa, resulting in decreased expression of the pro-inflammatory transcription factor NF-kB. Consistent with this concept, we found that heat treatment almost completely inhibited constitutive as well as radiation-induced activation of NF-κB in PC-3, and other prostate cell lines. Inhibition of this signal transduction pathway was independent of protein synthesis. Western analysis showed that IκBa expression was stabilized, as predicted.

The effect of irradiation on proteasome activity has been assessed using the same prostate cell lines. In all cases, irradiation resulted in a rapid drop in proteasome activity that recovered somewhat by 24 hours, but was still depressed. We have used purified proteasomes derived from the ECV304 cell line to show that this effect of radiation may be directly on both 26S and 20S activity and are repeating the experiments using proteasomes from prostate carcinoma cells. HSP90 does not appear to be the mechanism of inhibition, because geldamycin, a spcific inhibitor of HSP90 does not affect the outcome. Proteasome inhibition may be the prime mechanism by which radiation and heat rapidly up-regulate protein expression in cells, leading to an adaptive response with transcriptional activation of new genetic programs.

However, these findings raise a paradox. Several groups of investigators, including ourselves, have shown that radiation induces NF-kB expression. Our hypothesis would anticipate that radiation-induced inhibition of proteasome activity would decrease expression. We believe that we have resolved this issue by performing dose-response experiments. It seems radiation activates NF-kB only at high doses. At low doses, it increases IkBα expression and decreases NF-kB expression, as would be expected if proteasomedegradation is blocked. Seemingly, at high doses alternative pathways are activated to bypass proteasome inhibition. If these findings can be generalized to a number of different cell lines, one possible significance is that they provide mechanism for the anti-inflammatory effects of low dose radiation. Such effects have been observed in the clinic, especially during the first half of the last century, but are contradicted by the obviously pro-inflammatory nature of high dose exposure.

During this study another unexpected paradox came to light. We had expected that hyperthermia would stabilize expression of androgen recepetors, in keeping with blockage of the proteasome pathway that is responsible for their degradation. Unexpectedly, their expression decreased rapidly after heating. The mechanism is currently unknown, but may be related to disruption of the multimolecular complex within which the androgen receptors are bound intracellularly. In any event, this result could have clinical consequences, because we have shown that the surviving cells were able to proliferate in an androgen-independent manner, suggesting that heat may promote the transiton to androgen independence. These experiments again point to possible roles for proteasomes in regulating androgen dependence and independence.

Task 2. To identify pathways by which proteasome activity is altered in prostate cancer cells after hyperthermia and/or radiotherapy.

As we mentioned, our hypothesis is that heat-, but not radiation-, induced inhibition of proteasome activity is through activation of HSP90 which acxts as a natural inhibitor of proteasome activity. In experiments designed to test this concept, we have prepared the vectors encoding anti-sense HSP90, sense HSP90, and HSP70. We have not yet been able to obtain TRAP-1, but we have not direct evidence that this molecule is important in the response. These vectors will be used in prostate cancer cell lines to test our hypothesis and this will be the focus of this Task in the future.

Part of this task was to characterize the proteasome composition in prostate cancer cell lines. This has proved to be a more major task than we at first appreciated, requiring considerable preliminary efforts. Also, in the last year, evidence has accumulated that indicates more diversity in proteasome structure than was expected previously and our own data show differences between 20S and 26S with respect to heat-induced, but not radiation-induced inhition of proteasome activity, This necessitated a more detailed approach to the problem. In an attempt to deal with this complexity, we have successfully established gel assays for in situ assay of proteasome activity that can distinguish between 20S and 26S proteasome units and measure their function directly. This is now being exploited with heat and radiation treated cells. Our plan is to extend this to 2D gels to examine subunit expression, including LMP-2 and LMP-7.

We have also established cell lines that stably express Ub-GFP that can be regulated by proteasome inhibitors. These will enable us to study radiation- and heat-induced proteasome inhibition at a single cell level using flow cytometry and, hopefully, perhaps even the site of action within individual cells.

Task 3. To determine the extent to which 26s proteasome activity determines the effectiveness of prostate cancer treatment by hyperthermia and/or radiotherapy.

As stated above we have convincing data to indicate that hyperthermia inhibits proteasome degradation and prevents radiation-induced NF-kB activation. In many systems, NF-kB is a survival pathway. Inhibition of NF-kB could therefore increase the tendency to apoptosis and this could be one mechanism by which heat and radiation induce cell death. In addition, this mechanism could explain the radiosensitizing effect of heat given shortly prior to radiation treatment. Apoptotic responses and clonogenic survival are currently being tested under the same conditions to confirm that this is the case for prostate cancer cell lines.

Since we have shown that heat inhibits high-dose radiation-induced NF-kB expression, this could be the mechanism of radiosensitization. However, we have also some preliminary data using a dominant negative IkB adenoviral vector to suggest that, although NF-kB is a survival factor for many cell types, including prostate cancer, inhibition of NF-kB might not result in radiosensitization [Pajonk, 1999]. We are examining this pathway further. As an alternative hypothesis we proposed in the grant application a pathway by which proteasome inhibition might activate caspase 3 to degrade DNA-PKcs and inhibit DNA repair in cell lines after radiation and/or hyperthermia. We have investigated this and found that the timing of these effects is inconsistent with the hypothesis. The mechanism of heat-induced radiosensitization therefore still elusive. We have excluded a number of pathways, including p53. We still favor inhibtion of NF-kB as a mechanism, but it may not be the sole pathway, or even the most important one.

Key Research Accomplishments:

- Quantified proteasome function in prostate cancer cell lines
- Demonstrated inhibition of proteasome function in prostate cancer cells in response to hyperthermia
- Shown dependency of heat-induced proteasome inhibition on the 26S, but not 20S, subunit.
- Shown that heat-induced proteasome inhibition does not require protein synthesis.
- Shown temporal and temperature-dependent relationship between heating and HSP90 expression.
- Shown HSP90 dirctly inhibits proteasome function.
- Shown heat induces IkBa expression and decreases NF-kB expression

- Shown that radiation-induced proteasome inhibition results in increased IkBa expression and decreased NF-kB expression at low doses, but that this is circumvented at high doses, providing an explanation for the dual anti- and pro-inflammatory responses seen after irradiation.
- Established vectors for sense and anti-senseHSP90 and 70.
- Developed methodology for independently measuring 20S and 26S functional proteasome activity in gels.
- Developed methods for further purifying proteasomes on density gradients.
- Developed Ub-GFP stable transfectants that respond to proteasome inhibition.
- Shown that heat blocks high-dose radiation-induced NF-kB activation.
- Ruled out our hypothesis that heat-induced inhibition of proteasome activity leads to caspase3 activation, degradation of DNA-PKcs, and decreased DNA repair in response to ionizing radiation.

Reportable Outcomes:

Pajonk, F. and W.H. McBride: Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. Radiother. Oncol. 59:191-200, 2001.

Pajonk, F., C-S. Chiang, J-R. Sun and W.H. McBride: NF-kB, cytokines, proteasomes and low dose radiation exposure. Military Medicine, in press, 2001.

Pervan, M., F. Pajonk, J-R. Sun, H.R. Withers and W.H. McBride: The proteasome inhibitor PS-341 is a potential radiosensitizer. In: Abstracts for Proceedings of the Annual Meeting of the American Association for Cancer Research, 2001. (Presented at Annual Meeting 3/01.)

Pervan, M., F. Pajonk, J-R. Sun, H.R. Withers and W.H. McBride: Enhanced cellular radiation sensitivity upon proteasome inhibition. In: Abstracts for Proceedings of the Annual Meeting of the Radiation Research Society, 2001. (Presented at Annual Meeting 4/01.)

Conclusions:

This study is novel in that it investigates the effects of cancer therapy on protein expression resulting from inhibition of proteolytic degradation through the major cellular machinery, the proteasome. The mechanisms by which heat and radiation work to inhibit proteolysis appear to be different. This raises the question as to what subunits are affected and whether the diversity in proteasome activity between cancer cell lines is a reflection of differences in subunit structure. We will have to address this in more detail than was expected in the original proposal and new techniques are in development to examine this issue.

We have examined certain downstream effects of heat- and radiation-induced proteasome inhibition. A new, and unexpected, finding was that hyperthermia decreases androgen receptor expression resulting in androgen-dependent growth. This opens up a new line of investigation that will not be the focus of this study because it would divert us from our original intention, but its significance is obvious. As stated in the proposal, we have focused on how radiation- and heat-induced proteasome inhibition affects IkB expression, with concomitant decreases in NF-kB. NF-kB is a transcription factor that mediates most pro-inflammatory responses as well as acting as a survival factor for many cell types. The dose of radiation is important in determining the outcome. High doses are pro-inflammatory and able to circumvent proteasome inhibition to activate NF-kB, while low doses are anti-inflammatory. The significance of this is that it helps explain many clinical observations made over the last century. Heat treatment can block NF-kB in response to high dose radiation, providing a possible mechanism for heat-induced radiosensitization.

The long term significance of the knowledge gained from this study is that it, for the first time, presents the proteasome as a target for the action of radiation therapy and hyperthermia. It also indicates that variation in proteasome activity could determine the behavior of prostate cancer and their response to therapy. In the future, we predict that the proteasome will become a target for therapeutic intervention. We have already shown that drugs that affect proteasome function can act as radiosensitizers. One such drug, PS-341, is already in clinical trials in prostate, and other, cancers and is likely to be used in combination with radiation therapy in the near future.

References:

Pajonk, F, Pajonk, K, McBride, WH: Inhibition of NF-kB, clonogenicity, and radiosensitivity of human cancer cells. JNCI 91:1956-1960, 1999.

Tso, C-L., W.H. McBride, J-R. Sun, B. Patel, K-H. Tsui, S.H. Paik, B. Gitlitz, R. Caliliw, A. van Ophoven, L. Wu, J. deKernion and A. Belldegrun: Adrogen deprivation induces selective outgrowth of aggressive hormone refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. Cancer Journal, 6:220-223, 2000.

Appendices: None.